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TITLE: P53 Mutation Analysis to Predict Tumor Response in Patients Undergoing Neoadjuvant Treatment for Locally Advanced Breast Cancer

PRINCIPAL INVESTIGATOR: Lisa A. Carey, M.D.
Kathy Conway Dorsey, Ph.D.
Lynn Dressler, Ph.D.
Laura Esserman, M.D.
Michael Resnick, Ph.D.
Chad Livasy, M.D.
Charles Perou, Ph.D.
Michael Schell, Ph.D.
Scott Drouin
Brian Popko

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill, North Carolina 27599-1350

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Lisa A. Carey, M.D.; Kathy Conway Dorsey, Ph.D.;
Lynn Dressler, Ph.D., et al.

E-Mail: Lisa_Carey@med.unc.edu

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14. ABSTRACT

Studies suggest that p53 mediates responsiveness to chemotherapy . In an ongoing multiinstitutional prospective trial that is not supported by this award, breast cancer patients receiving neoadjuvant chemotherapy have serial response assessments and tumor sampling for research purposes. The project that is supported by this award involves analyzing the banked tumor specimens for p53 mutations using the GeneChip method, SSCP, and sequencing. We hypothesize that p53 status of the primary tumor will predict response to anthracycline-based and taxane-based chemotherapy given at different times in the same patient. A yeast-based functional assay is examining the impact of specific p53 mutations upon transactivation function. In the early years of the award, we optimized the GeneChip method of p53 mutation analysis for core biopsy specimens, successfully scaled down the DNA requirements allowing evaluation of small tumor biopsy samples, and optimized methods for p53 amplification within 1-2 large fragments so that SSCP and sequencing analysis were feasible despite the small amount of DNA available. P53 mutation analysis upon the study samples is now nearly complete. Implementation of the yeast-based functional assay for assessing the effect of specific p53 mutations has been successful with altered transactivation function found in mutations from neoadjuvantly treated patients.

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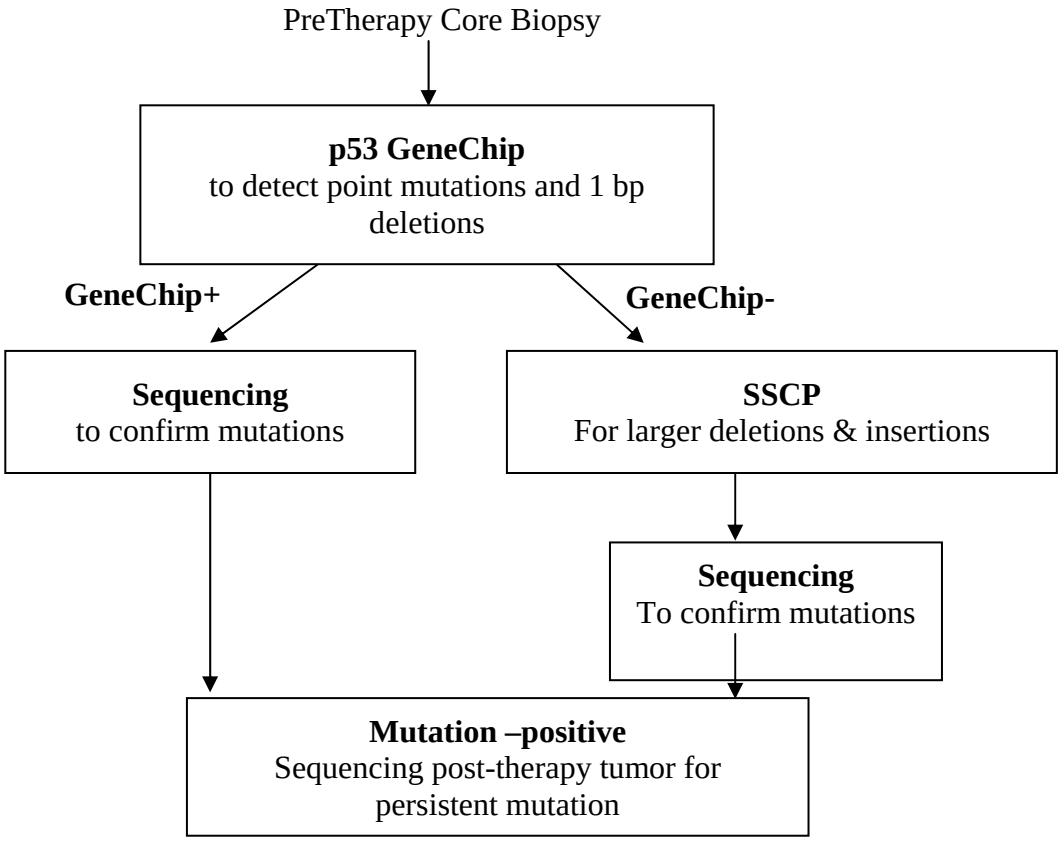
INTRODUCTION

The two most effective classes of chemotherapeutic drugs in breast cancer are the anthracyclines and the taxanes, which differ in mechanisms of action and resistance. Responsiveness to anthracyclines and taxanes may be mediated in part by the p53 mutational status of the tumor. P53 mutation status has had limited usefulness as a predictive tumor marker given the technical complexity of previous methods to determine it, however the development of p53 GeneChip technology has made high-throughput mutation analysis more feasible. This technology has been successfully applied to human tumor specimens [1,2]. Dr. Conway Dorsey's laboratory has previously determined the spectrum of expected p53 mutations in breast cancer[3] using sequencing, and is performing the GeneChip analysis and sequencing in this study.

In an ongoing multiinstitutional prospective trial, breast cancer patients who are receiving neoadjuvant chemotherapy have serial response assessments performed and undergo sampling of their tumor for research purposes at three time points. These timepoints are obtained prior to any chemotherapy and following treatment with an anthracycline-containing regimen. Those that receive a subsequent chemotherapy have another sample obtained after that regimen. This project involves analyzing the banked specimens for p53 mutation status using the GeneChip method. Specific mutations identified are further examined for functional impact in a yeast-based assay compared with the clinical response. We hypothesize that p53 status of the primary tumor predicts response to anthracycline-based and taxane-based chemotherapy given at different times in the same patient.

Our approach has been to first screen time point 1 (pre-therapy) cores by the Affymetrix p53 GeneChip microarray method, which detects point mutations and single base deletions, then to evaluate GeneChip-negative cores from time point 1 for larger deletions and insertions using single strand conformational polymorphism analysis (SSCP). All cores that are identified as potentially positive by the GeneChip or SSCP are then sequenced to confirm and specifically identify the mutation.

Mutation results are correlated with response to anthracycline and taxane chemotherapy. Dr. Resnick's laboratory has developed a yeast-based assay for identifying the functional consequence of specific mutations. Including the p53 sequences determined in neoadjuvantly treated breast cancer patients, they look for variable effects on transactivation function of p53 by mutation.



BODY

This award is for performance of laboratory assays upon banked tumor specimens obtained from ongoing correlative science trials that are funded through alternative mechanisms. The performance of those trials, however, is crucial to the outcome of this project, so is summarized here. The trials include Lineberger Comprehensive Cancer Center (LCCC) Project 9819 and a multiinstitutional trial, Cancer and Leukemia Group B (CALGB) Protocol 150007, which is a joint effort of CALGB, the American College of Radiology Imaging Network (ACRIN), and the National Cancer Institute Specialized Programs of Research Excellence (SPORE). These trials have accrued over 200 patients. All participating patients are required to have received an anthracycline-based chemotherapy and have ascertainment of clinical and pathologic response to therapy in order to be included. LCCC 9819 at this time has enrolled 59 patients and serves as a training set. CALGB 150007 limited access cooperative group trial completed accrual with 230 patients earlier this year. Of note, both LCCC 9819 and CALGB 150007 accrued a significant propotion, over 20%, of minority patients.

Statement of Work

Progress upon the approved statement of work is outlined below in the format used in the original application.

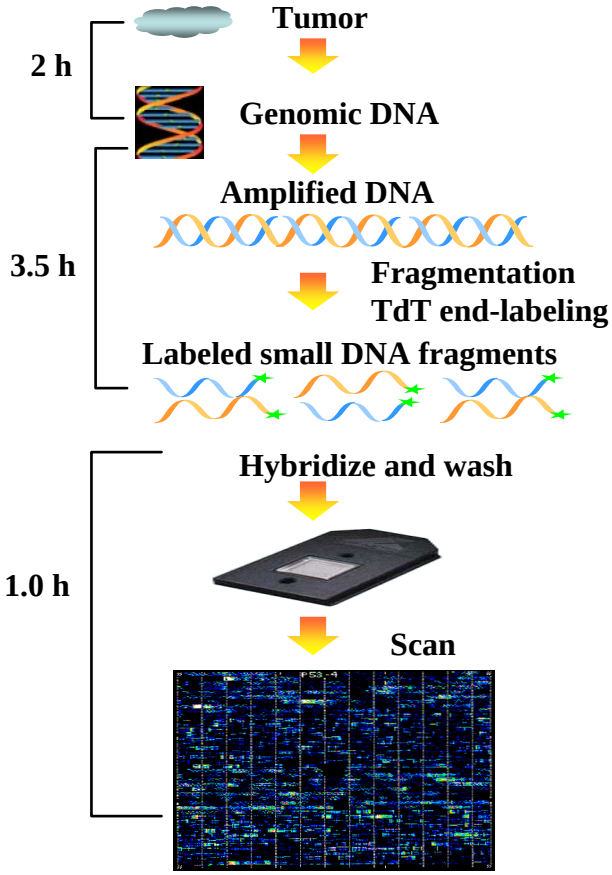
Task 1. To optimize the GeneChip method of p53 mutation analysis in the UNC Molecular

Epidemiology Core Laboratory (months 1-6)

Optimization of GeneChip method

This portion of the research involved the establishment of multiplex PCR conditions to co-amplify all p53 exons from within one reaction, and the optimization of the p53 GeneChip hybridization conditions and analysis of microarray data. Figure 1 illustrates the GeneChip method. The p53 GeneChip assay was optimized using the Affymetrix normal control DNA (human placental DNA) and cell lines (BT549, Bt474, MDA-MB-231, and MDA-MB-435), and was successfully applied to human breast cancer core biopsy specimens (Figure 2).

Figure 1. p53 GeneChip method



Because of the very small quantities of DNA obtained from the breast core biopsies, our first priority in establishing assay conditions for the p53 GeneChip assay was to determine the smallest amount of DNA that could be reasonably amplified from the cores, but that would provide a valid p53 mutation result. This is crucial because only 8-10mg of total tissue is obtained from each core biopsy, with nucleic acid-based studies planned by several collaborating laboratories. Using the above cell lines, the DNA concentration required for the multiplex p53 PCR reaction, which amplifies each p53 exon 2-11 in individual fragments, has been successfully reduced from an original amount of 250ng DNA down to 50ng DNA. This reduction of DNA has not compromised our ability to identify mutations within cell line DNA. The research plan includes single strand conformational polymorphism (SSCP) and sequencing analysis to comprehensively identify p53 mutations in GeneChip-negative samples. This technique of large-fragment PCR upon these limited tissue samples allows the SSCP and mutation analysis to be performed with a minimum of required DNA (Figure 3). The optimization of the GeneChip method was completed in 2003.

Task 2. To determine the p53 mutational status of the primary breast cancers before any

chemotherapy. In cases whose tumors exhibited p53 mutations pre-chemotherapy, determine if the same mutations are detectable after anthracycline then again after taxane with or without trastuzumab (Months 6-36).

Nucleic acids processing from core biopsies.

Dr. Conway-Dorsey and Dr. Perou are performing complementary assays (p53 mutation analysis and gene expression array, respectively) upon the frozen tissue in the UNC institutional trial. In the multiinstitutional trial, Dr. Joe Gray (UCSF) and Chris Haqq (UCSF) are also performing comparative genomic hybridization and additional RNA-based assays, so it has been crucial to optimize the nucleic acid retrieval method for this study. For this reason, a great deal of effort has been made to minimize the tissue, DNA, and RNA needs of each group so that all the planned assays may be performed. In order to optimize the conditions for maximal nucleic acid retrieval from these limited tissue resources, a training set of biopsies was obtained and tested. Several approaches to maximize RNA and DNA acquisition from core biopsies were tested, and we found that the optimal method remained the simplest. In this schema, tumor enrichment is performed by examination of an H&E-stained longitudinal section followed by manual dissection of non tumor-containing areas of the core. The remaining portion is divided evenly and one part is processed for RNA while the other part processed for DNA Prior to analysis of protocol core biopsies, 34 test core biopsies were successfully analyzed, providing DNA of good quality and, with only one exception, of adequate quantity.

Figure 2. Initial step GeneChip: Multiplex PCR amplification of exons 2-11 using 50ng DNA.

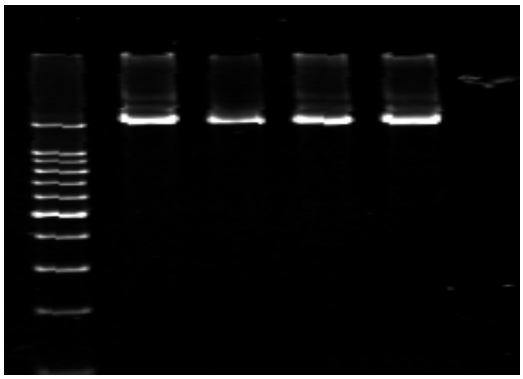
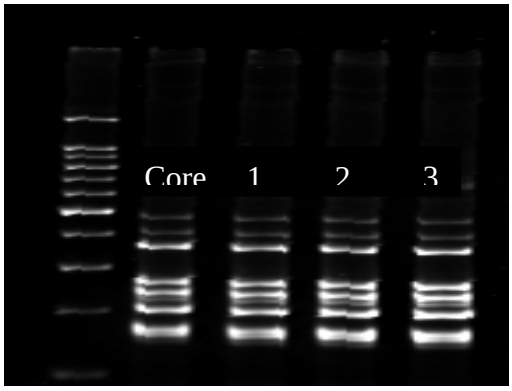


Figure 3. Initial step SSCP: PCR amplification of a single 1.5 kb fragment of p53 exons 4-8 using 50ng DNA.



The GeneChip assay is designed to detect point mutations and single base deletions in exons 2-11 of p53. After p53 Genechip microarray assays, tumors are then sequenced (for GeneChip-positive) or undergo SSCP (for GeneChip-negative).

We have received a total of 43 core breast biopsy DNA samples from the UNC institutional trial, LCCC 9819. DNA quality was adequate to successfully evaluate for p53 mutations in 42 of these 43 samples; only 1 sample failed to yield a PCR product. P53 gene mutations were identified in 20 of 42 (48%) of pre-therapy breast biopsies. Of the 20 mutations detected, there were 13 missense and 7 null mutations (3 frameshifts, 2 nonsense, 2 splicing). Mutations occurred most frequently in exons 5 and 8. Of the 20 mutations, 14 were initially detected by the p53 GeneChip array, and 6 more mutations were subsequently found by SSCP and/or sequencing. The mutations detected thus far are shown in Table 1. Seven of 26 (27%) core samples were p53 mutation-positive, with the following mutations being identified: R273H (hotspot), R283P, N239D (L3), R249S (L3, hotspot), D281G,

C141W, R213stop. As in the I-SPY/CALGB 150007 trial, the mutations were concentrated within the DNA-binding region, particularly within hotspot codons and the L3 loop domain.

Table 1. UNC institutional trial LCCC 9819 mutations

Mutation	Mutation Type	Exon/Intron
4bp del	frameshift	11
2bp del	frameshift	6
4bp del	frameshift	8
C141W	missense	5
H168R	missense	5
V172D	missense	5
H179R	missense	5
V173G	missense	5
V173G	missense	5
R213stop	nonsense	6
R196stop	nonsense	6
N239D	missense	7
R249S	missense	7
R249S	missense	7
R273H	missense	8
R283P	missense	8
D281G	missense	8
G266R	missense	8
nt12015,C>T	splicing	intron 3
intron g>a	splicing	intron9

We have also received a total of 243 DNA specimens from patients participating in CALGB 150007. Of these, 104 were from time point 1 core biopsies (pretherapy), 80 were from time point 2 biopsies (48-72 hours after initiation of anthracycline), and 59 were from surgical specimens (after completion of anthracycline and taxane). Our primary focus has been to evaluate p53 gene mutations in the pre-therapy (time point 1) cores. However, if a time point 1 DNA sample was not available, we analyzed the time point 2 biopsy (48-72 hours into treatment) and used this result as a proxy for time point 1. Using this approach, p53 mutational screening has been completed for 98 pretherapy, 43 time point 2, and 27 surgical samples, and p53 mutation-positive breast cancer was identified in 46 of 113 (40%) of patients. A small number of specimens (<10) that were recently received in the last shipment this summer have not yet been evaluated.

Combining the data from both studies, at this time 45% of the tumor samples have p53 mutations, most of which (75%) are missense, with a smaller proportion with null (22%) or silent (2%) mutations. Most are unique mutations, however mutations in hotspots have also been found: codon 273 (5, 2 in luminal B, 2 in basal-like, 1 unknown subtype), codon 249 (1 HER2+/ER-, 1 basal-like, 1 unclassified), codon 175 (4, all basal-like).

We have compared p53 mutational status between the time point 1 and time point 2 or surgical specimens as a means of validating the accuracy of our screening methods and gauging response to therapy. If the pre-therapy core biopsy was p53 mutation-positive, we evaluated the time point 2 and surgical samples to determine if the same mutation was still detectable during or after therapy. In 12 cases whose pre-therapy (time point 1) cores were p53 mutation-positive, the time point 2 samples tested were also positive for the same mutation. In some cases whose tumors were mutation-positive at time point 1, the mutation was not subsequently detected in the time point 2 or surgical specimen, presumably due to

tumor shrinkage in response to therapy and/or a low percentage of tumor in the core. In no instance did we detect a new or different p53 mutation in the time point 2 or surgical sample relative to the pretherapy specimen.

Task 3. To correlate p53 status with response to anthracycline chemotherapy, then taxane with or without trastuzumab in the same patient (months 30-36):

Clinical characteristics and response data are still being quality assured and entered into the databases used for this study, however preliminary characteristics are included in Table 2. Notably, the study populations are young, median age 45, 28% African-American, and high risk, with approximately 2/3 Stage III or IV.

Table 2. Clinical characteristics and chemotherapy regimen (modified Carey et al, ASCO 2006).

Median age	45
Race/ethnicity: white	65%
black	28%
other	6%
ER positive	53%
HER2 positive	21%
Pretreatment stage: II	36%
III	60%
IV	4%
Chemotherapy type:	
AC	13%
AC/taxane	64%
AC/taxane/trastuzumab	10%
Other	4%
Study: UNC 9819	43%
ISPY-1	57%
P53 status - wildtype	55%
- mutation	45%
Subtype - Luminal A	32%
- Luminal B	17%
- Basal-like	27%
- HER2+/ER-	20%
- Normal-like	2%
- unclassified	2%

We have conducted preliminary analyses to assess the relationship between p53 mutation status and response to neoadjuvant chemotherapy. From the initial test set, MRI-based response data was available after the anthracycline and taxane neoadjuvant therapy for 107 patients, and of these, we received DNA specimens for 94 patients. Within this group of 94 patients, 37 (39.4%) were p53 mutation-positive in the early pre-therapy or time point 2 cores. P53 mutation status may vary among response groups, with those exhibiting p53 mutation-positive tumors being more responsive than those with wildtype p53. P53 mutation-positivity in each group defined by response on MRI was: 50% (9/18) among complete responders (CR), 37% (19/52) among partial responders (PR), 42% (8/19) among those with stable disease (SD), and 20% (1/5) among those with progressive disease (PD). Taken in aggregate response categories, this became less clear - patients with wildtype or mutant p53 had similar MRI response overall; 74% (42/57) of those with wildtype p53 had CR or PR, and 76% (21/37) of p53 mutation-positive tumors had CR or PR.

Interestingly, it is possible that specific types of p53 mutations may affect response to therapy. CR or PR occurred among 88% (7/8) of patients with null mutations which produce a severely debilitated p53 protein, 100% (12/12) of patients with missense mutations at conserved residues, 100% (5/5) of patients with codon 175 missense mutations, and 85% (11/13) of patients with missense mutations in the L2 or L3 structural domains. In contrast, 60% (6/10) of patients with mutations at DNA or zinc binding residues showed SD or PD; 6 of these were missense mutations at codon 273.

Examining the pathologic samples revealed that 9 patients with p53 mutation-positive tumors had clinical CR to neoadjuvant therapy. Analysis of the surgical samples from 3 of

these patients with residual tumor showed that 1 carried the same mutation that was identified in the pretherapy core, while two had only wildtype p53. We did not receive surgical samples from the other patients. Among 8 patients with PR whose tumors were p53 mutation-positive, 6 had surgical samples that exhibited the same mutation as the pretherapy core, and two had only wildtype p53. Future studies will include completion of the p53 screening analyses for the remaining I-SPY 1 patients.

Previous studies have suggested that p53 mutation positivity may be associated with resistance to anthracyclines but enhanced response with taxanes. A core biopsy specimen was procured between administration of the anthracycline and the taxane for some patients, however these have not yet been processed for nucleic acids. Evaluation of inter-regimen (time point 3) cores may be helpful in separating out the effects of AC versus taxane.

Task 4. To compare p53 status with results of other planned assays within the larger correlative science trial such as bcl-2, estrogen receptor, and gene expression array analysis (months 1-36).

In a separately funded examination, molecular subtyping using gene expression array have been performed, revealing that approximately half of the patients are among the luminal subtypes, 27% basal-like, and 20% HER2+/ER-. Although these studies are not yet complete, at this time the proportion with p53 mutations differ by subtype (Table 3). Among those with both assays complete, we found that p53 mutations differed by subtype, with Basal-like cancers the most frequent (79%), then HER2+/ER- (44%), Luminal B (36%), and Luminal A (21%) ($P<0.001$).

Table 3. GeneChip p53 results by gene expression array subtype from the pretreatment sample from ISPY-1/CALGB 15007 training set plus UNC 9819.

Subtype	P53 mutant	P53 wildtype	Total
Luminal A	7 (21%)	27 (79%)	34 (34%)
Luminal B	5 (36%)	9 (64%)	14 (14%)
Basal-like	23 (79%)	6 (21%)	29 (29%)
Normal-like	0	2 (100%)	2 (2%)
Unclassified	2 (67%)	1 (33%)	3 (3%)
Total	45 (45%)	54 (55%)	100

This is consistent with our results from the p53 mutation analysis of the population-based Carolina Breast Cancer Study, in which p53 mutations were significantly more frequent among Basal-like (44%) and HER2 (43%), while Luminal B (23%) and Luminal A (15%) had fewer mutations (Carey, JAMA 2006). As has been previously demonstrated, this has implications for chemosensitivity, and further examination of the interaction between subtype and p53 mutation status regarding response to therapy will be performed.

Other analyses, including immunohistochemistry for apoptosis and cell cell cycle markers, cd34 for angiogenesis, and proteomics, are in progress. The NCI has developed an

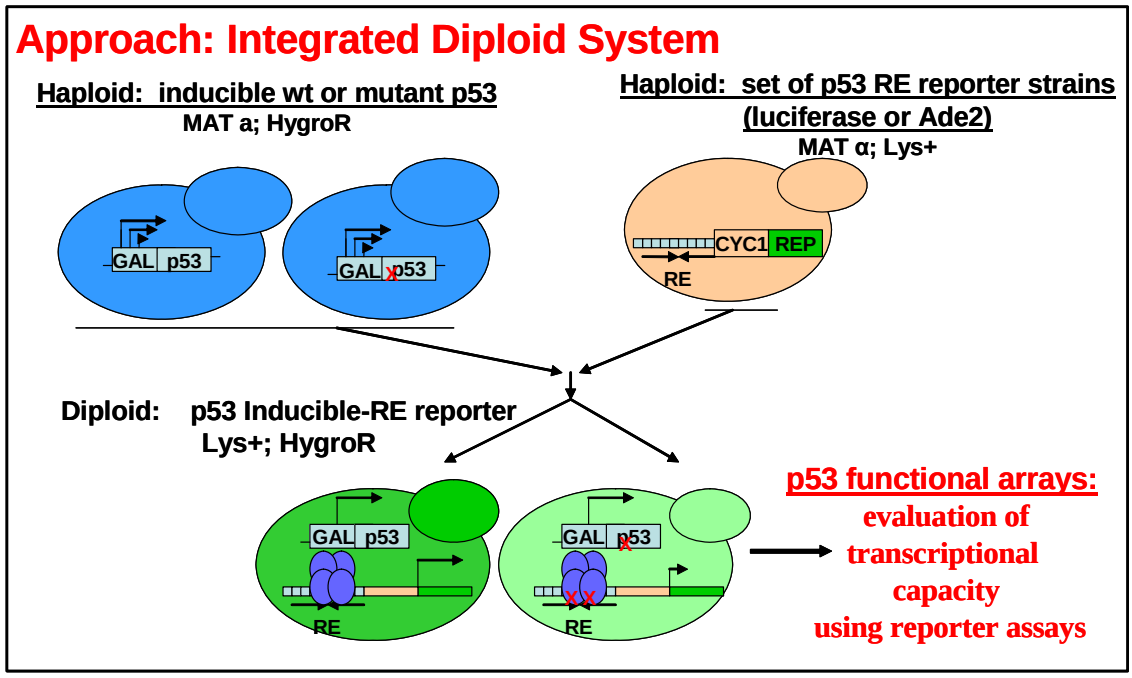
integrated database for analysis of these markers, which will be performed in the upcoming year now that accrual to CALGB 150007 is complete.

Task 5. To functionally classify the p53 mutants identified in breast cancer using established and newly developed yeast-based assays (Months 12-36).

Establish system for functional characterization of various p53 mutations associated with breast cancer.

Twenty-three distinct p53 missense mutations were found in breast cancer patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer. A yeast model system was utilized to analyze the functional consequences of these p53 missense mutations towards many p53 response elements (REs) derived from human genes (Figure 4). Evaluation of transactivation capacity was based on a qualitative, visual assay using an *ADE2* color system and a quantitative luciferase reporter. Both reporters exploit a "rheostatable" promoter for p53 expression and utilize the "*delitto perfetto" in vivo* mutagenesis approach for rapid inclusion of REs upstream of a reporter and the development of mutant p53s (Inga, Monti et al. 2001; Inga and Resnick 2001; Storici, Lewis et al. 2001; Inga, Nahari et al. 2002; Inga, Storici et al. 2002). This system compares p53 variants at variable expression levels in a constant, isogenic chromatin environment.

Figure 4. Determining mutant p53 transactivation potentials. Transactivation capacity of p53 is evaluated using diploid cells where both p53 and human target response elements (REs) are integrated into specific chromosomal loci. Two panels of modified S. cerevisiae strains are generated. The first is a set of "p53-host" strains in which p53 (wildtype or mutant) is controlled by a "rheostatable" GAL 1,10 promoter. The second contains promoter REs upstream of the either the *Ade2* or firefly luciferase reporter. Mating of these strains results in isogenic, isogenomic diploid yeast that enable the assessment of the transactivation potential for wt or mutant p53 proteins towards individual REs in the p53 transcriptional network. Each strain differs only by the mutation of interest and the 4-5 bases a particular RE varies from the consensus.



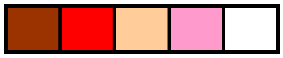
Twenty-one p53 missense mutations found in patient core biopsies have been studied to date in the *ADE2*-based visual reporter system using twelve different REs associated with a variety of p53-dependent biological responses. Fifteen of the twenty-one mutations were classified as loss-of-function mutations based on the inability to transactivate the reporter from any RE. Similarly, functional analysis of mutants in IARC database also indicated that the mutants in the present neoadjuvant study are predominantly loss-of-transactivation. However, among the 21 p53 missense mutants that included hotspot and non-hotspot residues, 6 showed altered functions--not complete loss--towards at least one RE (Table 4).

Table 4. P53 missense mutations associated with breast cancers retain function. Twenty-one p53 missense mutations identified from tumor core biopsies in patients undergoing neoadjuvant chemotherapy have been assessed for functionality in the isogenic, *in vivo* yeast system. Six of the twenty-one (~29%) p53 missense mutations analyzed were shown to have altered transactivation capacity in comparison to wt p53 towards 13 response elements (REs) associated with human downstream target genes.

	IARC DATABASE * Total mutations			
p53 missense mutation	Somatic [breast tumors]		Germline	Functional Status
L130V	15	[2]	0	**ALTERED**
C141W	11	[0]	0	**ALTERED**
P151H	31	[4]	0	LOSS
G154S	10	[0]	0	LOSS
V173L	77	[10]	0	LOSS
R175H	hotspot	hotspot	hotspot	LOSS
Y220C	263	[35]	17	**ALTERED**
N239D	36	[5]	0	LOSS
C242Y	44	[5]	10	LOSS
G245S	343	[33]	51	LOSS
M246A	0	0	0	LOSS
R248L	hotspot	hotspot	0	LOSS
R249S	351	[13]	0	LOSS
D259V	16	[2]	0	LOSS
G266R	61	[5]	0	LOSS
R273C	hotspot	hotspot	hotspot	LOSS
R273H	hotspot	hotspot	hotspot	LOSS
P278A	19	[4]	0	**ALTERED**
D281G	14	[3]	0	LOSS
R283P	31	[3]	0	**ALTERED**
E285K	139	[20]	0	**ALTERED**

Interestingly, at high levels of p53 expression, three of the six mutations that retain function (L130V, C141W, E285K), look similar to wt p53 in their transactivation capacity (Figure 5). Reducing the expression of p53 (by decreasing galactose in the media) revealed subtle

transactivation defects in these mutations that were masked at higher expression thus differentiating themselves from wt p53 and other p53 missense mutations (Figure 5). Thus, all the p53 missense mutations with retained function examined have an impact on function. However, for some mutations the impact on the transcriptional network or biological response may occur only at low levels of p53 expression.

Figure 5. Altered function p53 missense mutations associated with breast cancers have subtle effects that are exaggerated at low levels of p53. The functional fingerprints of individual p53 missense mutations were determined by assessing their ability to transactivate from 13 REs using the qualitative Ade2 plate assay. This phenotypic color assay determines the ability of p53 variants to transactivate from specific REs in stationary cells through the accumulation of pigment. If p53 (wt or mutant) is capable of strongly transactivating from a RE, colonies are white, whereas, if p53 is not able to transactivate from a RE, colonies are red. Variation in color correlates to different magnitudes of transactivation. (transactivation: weak  strong)

0.128% galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

0.064%galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

0.032% galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

0.016% galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

0.008% galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

0.004% galactose

RE / Allele	con A	P21	P53R2	GADD45	14-3-3σ	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A+B
WT													
L130V													
C141W													
Y220C													
P278A													
R283P													
E285K													

The quantitative luciferase-based assay is also being used to characterize the altered function mutants in greater detail. Preliminary results with the quantitative assay showed altered function p53 missense mutations associated with breast cancers reduce the levels of transactivation *in vivo* from specific REs (Figure 6). The induction patterns were consistent with the functional mutation (L130V) simply modulating transactivation levels in comparison to wt p53. As expected, the loss-of-function mutation (G154S) shows an inability to transactivate from the RE. Mutations that are found to have an altered function will be further examined by the quantitative luciferase assay to determine how the specific mutation affects the "kinetics" of transactivation towards multiple REs.

Figure 6. Altered function missense mutations reduce the level of transactivation *in vivo*. The quantitative luciferase reporter was used to follow the "kinetics" of transactivation for wt and variant p53 over a range of p53 expression levels. Preliminary results from the quantitative luciferase assay show that altered function p53 missense mutations (L130V) have a reduced capacity to transactivate from specific RE sequences in comparison to wt p53, whereas loss-of-function mutations (G154S) have lost the ability to transactivate from REs.

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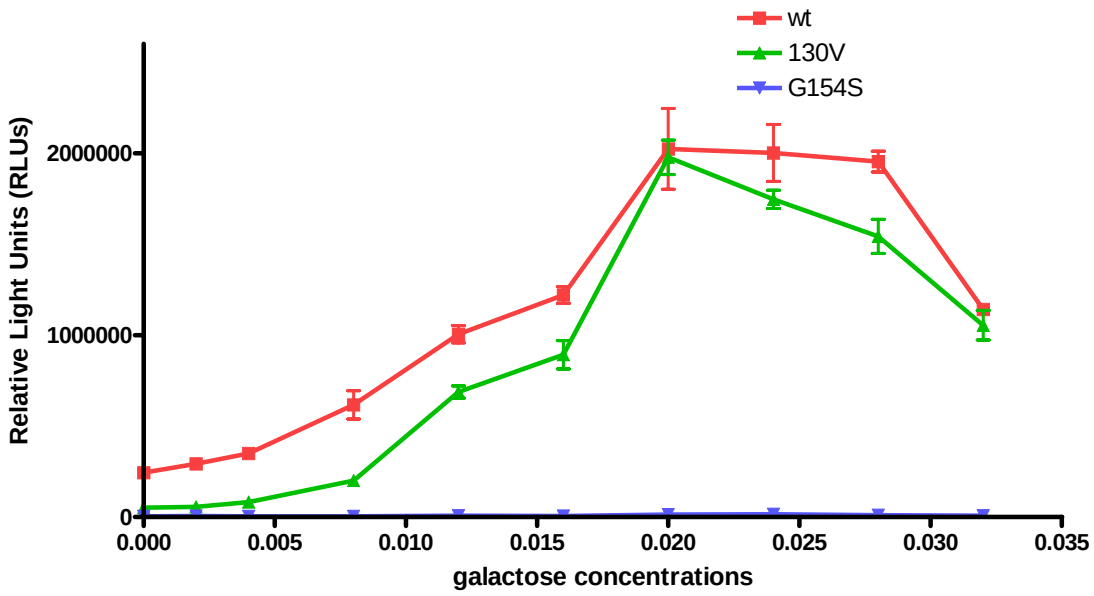

Given that many non hotspot p53 mutations associated with cancer retain function as indicated in Table 4, these results for the breast cancers in the neoadjuvant study are unexpected and may reflect a clinical feature of the breast cancers examined. Consistent with the findings of a shift in the nature of the p53 mutations seen in this population compared with the Carolina Breast Cancer Study in that the present study has more missense mutations, we may be seeing a trend for more functionally altered p53 mutations among these highly-selected large, possibly more rapidly growing tumors. p53 mutations occurring in these tumors may be more deleterious and reflect alterations in the transcriptional regulatory activity of p53.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized conditions for GeneChip assay using frozen breast cancer tissue
- Scaled down DNA requirements for GeneChip assay to require only 50ng DNA.
- Optimized method of concurrent RNA and DNA processing from core biopsies.
- Optimized large, single fragment PCR reaction to amplify entire coding region of p53 from large molecular weight DNA
- Optimized SSCP/sequencing methods for additional exons 2, 3, 9, 10, and 11 in addition to the existing protocol for exons 4-8.
- Identified positive control cell lines for SSCP that contain known p53 mutations in each of exons 2, 3, 9, 10, and 11.
- Adapted a system for in vivo site-directed mutagenesis by oligonucleotides to allow rapid construction in yeast of any p53 mutant.
- Developed seven isogenic p53 host yeast strains that can be used to generate any p53 mutation between amino acid 118 and 329
- Developed method to mate p53 host strain with p53 reporter strain allowing characterization of functional impact of chosen p53 mutation
- Collected clinical data, response to therapy data, and DNA from breast cancer tissue before, during, and after anthracycline- followed by taxane-based chemotherapy in an institutional pilot trial.
- Collected clinical data, response to therapy data, and DNA from breast cancer before, during, and after anthracycline-based chemotherapy in a multicenter trial that recently completed accrual.
- Comprehensive p53 mutation status determined from the tumors from patients in the institutional trial, and 113 tumors from patients in the multicenter trial has revealed a higher proportion than expected (45%) with p53 mutations.
- Most (78%) were missense mutations, which can have variable impact upon p53 function.
- There were significant differences between the molecular subtypes in proportion with p53 mutations, with the highest among Basal-like breast cancers (79%) and the lowest among Luminal A breast cancers (21%).
- Ongoing collaborations with investigators performing gene expression array (Perou) and aCGH (Gray) will allow further examination of p53 mutation type with breast cancer subtype and gene copy number abnormalities.
- Many non hotspot p53 mutations identified in this study reveal variable transactivation functions, which may impact upon p53 effect on clinical endpoints.

REPORTABLE OUTCOMES:

- P53 mutational spectra by gene expression subtype in neoadjuvantly treated breast cancer patients in a multicenter cohort (Carey LA et al, ASCO 2006)
- Identification of functional p53 mutants that affect the kinetics and spectrum of transactivation in vivo that are found in breast cancer. (Jordan JJ et al, DOD Annual Meeting 2005; Jordan JJ et al, AACR 2005)
- Determination of optimal method to measure response to neoadjuvant chemotherapy in clinical / translational trials (Metzger R et al. JNCI, 2005)
- Correlation of primary tumor chemosensitivity by hormone receptor and HER2 status and preliminary evaluation of p53 by receptor phenotype (Carey LA et al, Breast Cancer Res Treat 78 (S1):1023a, 2004
- Correlation of p53 by immunohistochemistry and HER2 status with response to anthracycline- then taxane-based chemotherapy (Carey LA et al, Breast Cancer Res Treat 78 (S1):1033a, 2004)
- In vivo site-directed mutagenesis system adapted to allow rapid construction of defined p53 mutants in yeast (Storici, Durham et al. PNAS, 2003)
- Method of characterization of p53 alleles with altered transactivation function (Inga, Storici et al. Mol Cell Biol 2003; Resnick and Inga, PNAS 2003)

CONCLUSIONS

Ascertainment of tumor samples from patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer is continuing. In addition to the UNC samples, the NCI-supported multiinstitutional study began in the fall 2002, has recently completed accrual. The planned analyses of p53 as a predictive marker in breast cancer are well underway. Preliminary data from from the UNC instititutional pilot trial suggests that there is likely to be an 80% response rate to the neoadjuvant chemotherapy administered to patients in the UNC institutional trial and the CALGB 150007 multiinstitutional study. The gene expression array and array-CGH correlates to the p53 mutation analysis being performed under this award are also ongoing, preliminary analyses of gene expression arrays suggest an interaction between breast cancer subtype and p53 loss supporting the completion of these studies.

The GeneChip method of p53 mutation analysis in human tumors has been optimized in Dr. Conway Dorsey's laboratory. Moreover, her laboratory has successfully reduced the required amount of DNA to 50 ng, and has performed multiplex PCR amplification upon both test and study core biopsies with good results. The screening method for GeneChip-negative samples using SSCP and sequencing has been optimized for the entire coding region of p53 from the large molecular weight DNA obtained from core biopsies, and have identified positive cell line controls for mutations in each exon. Preliminary analyses of the data from the multiinstitutional trial suggests that p53 mutations are frequent among the large tumors included in this trial, and that missense mutations are more frequent than expected based on previous studies in smaller tumors. The correlation of p53 status with clinical endpoints such as response to therapy awaits further clinical and pathologic response data from the cooperative group trial.

Once mutations are identified in the tumors from patients undergoing neoadjuvant chemotherapy, the specific mutations are provided to Dr. Resnick in order to correlate clinical response to therapy with functional evaluation of the effect of these mutations upon transactivation in a yeast-based transactivation assay. Dr. Resnick's laboratory has adapted their in vivo site-directed mutagenesis method to allow rapid construction of p53 mutations of choice. They have also demonstrated the ability to characterize p53 alleles with altered transactivation functions. Of mutations from tumors in patients treated on the neoadjuvant trials, most were loss-of-function mutations, however some variable transactivation function loss has been seen, with clinical significance to be determined in this study. The functional fingerprints remain to be determined for the remainder of the identified missense mutations.

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